Effect of Bevacizumab on Inflammation and Proliferation in Human Choroidal Neovascularization

Olcay Tatar, MD; Efdal Yoeruek, MD; Peter Szurman, MD; Karl Ulrich Bartz-Schmidt, MD; for the Tübingen Bevacizumab Study Group; Annemarie Adam, MTA; Kei Shinoda, MD, PhD; Claus Eckardt, MD; Vicky Boeyden, MD; Carl Claes, MD; Grazia Pertile, MD; Gabor B. Scharioth, MD; Salvatore Grisanti, MD

Objective: To evaluate the effect of bevacizumab (Avastin; Genentech, Inc, South San Francisco, California) on inflammation and proliferation in human choroidal neovascularization (CNV) secondary to age-related macular degeneration.

Methods: Retrospective review of interventional series of 38 patients who underwent choroidal neovascular membrane (CNVM) extraction. Twenty-four patients received intravitreal bevacizumab 1 to 154 days preoperatively (bevacizumab CNV group). Fourteen patients received no preoperative therapy (control CNV group). The CNVM were stained for cytokeratin 18, CD68, CD45, intercellular adhesion molecule (ICAM)–1, E-selectin, Ki-67, Thy-1, and endostatin.

Results: No significant difference was detected in ICAM-1 and E-selectin expression between groups. The density of leukocytes in the bevacizumab CNV group (median, 271.61 cells/mm²) was higher than in the control CNV group (median, 116.87 cells/mm²; \( P = .07 \)), but without significance. Density of macrophages (median, 4661.95 cells/mm²), proliferative activity (median, 160.19 cells/mm²), and percentage of Thy-1–expressing vessels (median, 100%) were significantly higher in the bevacizumab CNV group than in the control CNV group (median, 882.66 cells/mm², \( P < .001 \); median, 34.34 cells/mm², \( P < .001 \); and median, 80%, \( P < .001 \), respectively). Endostatin immunoreactivity was considerably stronger in the retina pigment epithelium (RPE)–Bruch membrane complex (median, 3; range, 2-3; \( P < .001 \)), and stroma (median, 3; range, 1-3; \( P < .001 \)) of the bevacizumab CNV group than control CNV group (median, 1.5; range, 0-3 and median, 1; range, 0-3, respectively).

Conclusions: Unexpectedly, CNVM from patients treated by bevacizumab are characterized by significantly high inflammatory and proliferative activity and enhanced endostatin expression. These characteristics need to be considered when protocols for combination therapies are established.

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A GE-RELATED MACULAR DEGENERATION (AMD) is the leading cause of legal blindness in patients older than 60 years in the Western world. Neovascular AMD with the development of a choroidal neovascularization (CNV) accounts for 80% of the severe loss of visual acuity due to AMD.

Introduction of angiogenesis inhibitory drugs, especially of vascular endothelial growth factor (VEGF) inhibitors, has been an important milestone in neovascular AMD treatment and has limited the indications for other treatment modalities considerably.

Of the VEGF inhibitors, pegaptanib (Macugen; Eyetech Pharmaceuticals, New York, New York) and ranibizumab (Lucentis; Genentech, Inc, South San Francisco, California) were shown to be effective in the treatment of neovascular AMD in large clinical trials. Recently, bevacizumab (Avastin; Genentech, Inc), a full-length recombinant humanized monoclonal antibody against VEGF used previously for the treatment of metastatic colorectal cancer, has been introduced to ophthalmology. Use of bevacizumab either as a monotherapy or as an adjuvant to photodynamic therapy (PDT) seems promising in CNV treatment. However, insight into the effect of these anti-VEGF drugs on human CNV is still limited.

Herein, we present our results of a clinicopathological evaluation of surgically excised choroidal neovascular membranes (CNVM) treated with an intravitreal bevacizumab injection prior to surgery. Our study focuses on inflammation and proliferative activity. Inflammatory infiltration was evaluated using antibodies against CD68 and CD45 (common leukocyte antigen) as markers for macrophages and leukocytes, respectively. Considering their involvement in the recruitment of inflammatory cells, expression of intercellular adhesion molecule (ICAM)–1 and E-selectin were also evaluated. The

Author Affiliations are listed at the end of this article.
investigation included Thy-1, owing to its characteristics as a cell surface marker expressed on vascular endothelial cells (EC) that are upregulated by the inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α. Endostatin is an endogenous angiogenesis inhibitor that is activated by proteases such as those derived from inflammatory cells. The antigen Ki-67 was used as a nuclear marker of cell proliferation.

**METHODS**

**PATIENTS AND TREATMENTS**

We retrospectively reviewed 38 eyes of 38 consecutive AMD patients in different centers who were surgically treated with full macular translocation surgery with CNVM extraction. All patients had clinically active disease and progression. Fourteen CNV patients without any kind of therapy prior to surgery composed the control CNV group. The clinical characteristics of these 14 patients are summarized in Table 1. Twenty-four patients received surgery following intravitreal bevacizumab injection (bevacizumab CNV group). Therapy options, including observation, conventional thermal laser photocoagulation, verteporfin PDT, intravitreal injection of triamcinolone acetonide or anti-VEGF agents, and full macular translocation with 360° retinotomy and CNVM extraction were discussed with the patients. Although submacular removal of CNV was not favored by the randomized submacular surgery trials, submacular surgery trial Group B revealed that submacular surgery for predominantly hemorrhagic CNV did reduce the risk of severe visual acuity loss (loss of ≥6 Early Treatment Retinopathy Study lines) and contrast sensitivity loss (≥3 segments, using the Pelli-Robson chart at 0.5 m) significantly, in comparison to the group observed without any treatment for 24 months of follow-up. Macular translocation of the retina, a complex but potentially effective surgical method, was reported to be beneficial when CNV was related to submacular hemorrhage or retinal tear. Furthermore, improvement in distance and near visual acuity as well as in reading speed was detected at the 1-year follow-up of patients with neovascular AMD who were treated with macular translocation.

Intravitreal bevacizumab injection and surgical intervention with full macular translocation surgery were offered when (1) visual acuity was worse than 20/200 (being the minimum visual acuity to recommend the first PDT, according to the Treatment of Age-Related Macular Degeneration with Photodynamic Therapy investigation); (2) CNV was related to retina pigment epithelium (RPE) tear, chorioretinal anastomosis, or massive subretinal/intravitreal hemorrhage; (3) visual deterioration progressed and subretinal hemorrhage and RPE tear appeared following treatment; or (4) reading ability was recently lost, despite improvement in distant visual acuity after previous treatment. The clinical characteristics of the patients treated with intravitreal bevacizumab are summarized in Table 2. Reduction of intraoperative bleeding was intended with the preoperative injection of bevacizumab, as suggested in other ocular pathologies.

Each patient gave written informed consent regarding their knowledge of the off-label use and experimental nature of the treatment procedure, and the risks and benefits of all therapeutic options were discussed in detail. The study followed the guidelines of the declaration of Helsinki. Local institutional review board approval was granted for allocation and histological analysis of the specimens.

**TISSUE PREPARATION AND IMMUNOHISTOLOGY**

Within minutes after surgery, excised CNVM were fixed in 3.7% formalin and embedded in paraffin. After serial sections were de-paraffinized and rehydrated, antigen retrieval was accomplished by proteolytic digestion with 0.5% protease XXIV (Sigma, St Louis, Missouri) for cytokeratin 18, endostatin, and Thy-1, and by heat treatment in citrate buffer in a pressure cooker under 120°C for 2 minutes for Ki-67, CD45, CD68, ICAM-1, and E-selectin. Immunohistochemical staining with the primary mouse monoclonal antibodies specific for cytokeratin 18 (Progen, Heidelberg, Germany), ICAM-1 (Novocastra, Newcastle upon Tyne, England), E-selectin (Novocasta), CD45 (Dako, Glostrup, Denmark), and Ki-67 (Clone Ki-55; Dako) was performed using the avidin-biotin complex horseradish peroxidase method, as previously described. For E-selectin, cytokeratin 18, and ICAM-1 staining, the brown chromogen 3-diaminobenzidine was replaced with 3-amino-9-ethylcarbazole, a highly sensitive substrate chromogen (Code K3461; Dako).

Immunohistochemical staining for Thy-1, CD68, and endostatin was performed by the alkaline-phosphatase method, according to the manufacturer’s instructions (ChemMate Detection Kit, Alkaline Phosphatase/RED Rabbit/Mouse, K3005; Dako), as previously described, using primary antihuman antibodies specific for Thy-1 (mouse anti-CD90 antibody; BD Biosciences Pharmingen, San Jose, California), CD68 (mouse, monoclonal; Dako), and endostatin (rabbit, polyclonal; Dianova GmbH, Hamburg, Germany). Color was developed using chromogen red (ChemMate Detection Kit; Dako). Hematoxylin (Chemmate, Code S2020; Dako) was used for counterstaining.

For negative controls, primary antibodies were substituted by the appropriate normal sera or omitted.

**ANALYSIS**

Serial sections from a specimen were analyzed by light microscopy. Immunoreactivity for ICAM-1, E-selectin, CD68, and endostatin was analyzed semiquantitatively and separately in the RPE, EC (in the perivascular area for CD68), and stroma. A grading scheme indicating the degree of labeling was used: 0, 1, 2, and 3 were assigned for 0%, 1% to 39%, 40% to 69%, and 70% to 100% positive cells, respectively. Absence of staining was scored 0.

Each specimen was documented under ×50 magnification with a Zeiss microscope (Axioskop; Carl Zeiss, Inc, Oberkochen, Germany) connected to a digital camera (Fujix HC300Z; Fujifilm, Tokyo, Japan). The area of each specimen was measured using the appropriate hardware and software (Axiovision, Version 3.1; Carl Zeiss, Inc, Gottingen, Germany).

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**Table 1. Clinical Characteristics of Patients Without Any Kind of Preoperative Therapy**

<table>
<thead>
<tr>
<th>Patient, No./</th>
<th>Sex/Age, y</th>
<th>Eye</th>
<th>CNV Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/F/71</td>
<td></td>
<td>Right</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>2/F/80</td>
<td></td>
<td>Right</td>
<td>Occult with hemorrhage</td>
</tr>
<tr>
<td>3/F/75</td>
<td></td>
<td>Left</td>
<td>Predominantly classic</td>
</tr>
<tr>
<td>4/M/75</td>
<td></td>
<td>Right</td>
<td>Classic with hemorrhage</td>
</tr>
<tr>
<td>5/F/75</td>
<td></td>
<td>Right</td>
<td>Classic with hemorrhage</td>
</tr>
<tr>
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<td></td>
<td>Right</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>7/M/80</td>
<td></td>
<td>Left</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>8/M/80</td>
<td></td>
<td>Left</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>9/M/72</td>
<td></td>
<td>Left</td>
<td>Classic with hemorrhage</td>
</tr>
<tr>
<td>10/F/73</td>
<td></td>
<td>Right</td>
<td>Hemorrhagic</td>
</tr>
<tr>
<td>11/F/74</td>
<td></td>
<td>Left</td>
<td>Occult with hemorrhage</td>
</tr>
<tr>
<td>12/F/81</td>
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<td>Right</td>
<td>Hemorrhagic</td>
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<td>Hemorrhagic</td>
</tr>
<tr>
<td>14/M/64</td>
<td></td>
<td>Right</td>
<td>Occult</td>
</tr>
</tbody>
</table>

Abbreviation: CNV, choroidal neovascularization.
All Ki-67–positive nuclei, macrophages, and leukocytes were counted in each specimen. Proliferative activity, density of leukocytes, and macrophage infiltration in each specimen were determined quantitatively by calculating the ratio of the total number of Ki-67 positive nuclei, macrophages, and leukocytes in the CNVM to the total area of the membrane.

Thy-1 expression was determined by the percentage of Thy-1–expressing vessels in the overall vascularization of each membrane.

Intensity of CD68, ICAM-1, E-selectin, and endostatin immunostaining, density of leukocyte and macrophage infiltration, proliferative activity, and Thy-1 expression in the described CNV groups were compared using the Mann-Whitney U test. Immunohistologic findings in the bevacizumab CNV group were analyzed based on timing or number of bevacizumab injections, using the Spearman correlation test. A P value less than .05 was considered significant.

RESULTS

Immunohistological findings are summarized in Figure 1.

HISTOPATHOLOGICAL FINDINGS IN THE CONTROL CNV GROUP

Retina pigment epithelial cells immunoreactive for cytokeratin 18 were found in all specimens. All Ki-67–positive nuclei, macrophages, and leukocytes were counted in each specimen. Proliferative activity, density of leukocytes, and macrophage infiltration in each specimen were determined quantitatively by calculating the ratio of the total number of Ki-67 positive nuclei, macrophages, and leukocytes in the CNVM to the total area of the membrane. Thy-1 expression was determined by the percentage of Thy-1–expressing vessels in the overall vascularization of each membrane. Intensity of CD68, ICAM-1, E-selectin, and endostatin immunostaining, density of leukocyte and macrophage infiltration, proliferative activity, and Thy-1 expression in the described CNV groups were compared using the Mann-Whitney U test. Immunohistologic findings in the bevacizumab CNV group were analyzed based on timing or number of bevacizumab injections, using the Spearman correlation test. A P value less than .05 was considered significant.

Expression of E-selectin was found in the RPE of all CNVM, and intensely in 4 (28.6%) membranes. E-selectin was detected in the EC of 8 CNVM (57.1%) and in the stroma of 9 CNVM (64.3%) (Figures 1A and 2B). Expression of ICAM-1 was prominent in the RPE; it was intense in 13 CNVM (92.9%). No ICAM-1 was present in the EC of 9 CNVM (64.3%). Intense ICAM-1 expression was detected in the EC of 3 (21.4%) and in the stromal cells of 2 CNVM (14.3%) (Figures 1B and 2C). Leukocytes detected by CD45 immunostaining were found to be present in all but 1 CNVM. The number of leukocytes in a 1-mm² area of CNVM varied between 0 and 779.6 (median, 106.64 cells/mm²) (Figure 1C). The specimen in Figure 2D has the fourth highest density of leukocyte infiltration (157.39 cells/mm²) in the control CNV group.

The density of macrophages ranged between 183.94 cells/mm² and 2826.73 cells/mm² (median, 882.66 cells/mm²) (Figure 1C). Macrophage infiltration was seen in the RPE cell layer, the perivascular area, and the stroma in 85.7% (n=14), 61.5% (n=13), and 92.8% (n=14) of the specimens. Intense macrophage infiltration was detected in the RPE cell layer of 6 (42.8%), the perivascular area of 2 (14.3%), and the stroma of 4 (28.6%) CNVM (Figures 1D and 2E).

Vessels expressing Thy-1 were detected in 13 of 14 specimens (92.8%), in varying percentages of vessels (median, 80.0%; range, 0%-100%). In 4 (28.6%) CNVM, all vessels displayed Thy-1 (Figure 2F).

Table 2. Clinical Characteristics of Patients Treated With Intravitreal Bevacizumab Before Submacular Surgery

<table>
<thead>
<tr>
<th>Patient, No./Sex/Age, y</th>
<th>Eye</th>
<th>CNV Type</th>
<th>No. of Bevacizum Injections</th>
<th>Time to Surgery From Treatment, d</th>
<th>PDT</th>
<th>Intravitreal Bevacizum</th>
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</thead>
<tbody>
<tr>
<td>1/F/80</td>
<td>Left</td>
<td>Hemorrhagic</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>2/F/66</td>
<td>Left</td>
<td>Hemorrhagic</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>3/M/75</td>
<td>Right</td>
<td>Occult hemorrhagic with RPE tear</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>4/M/69</td>
<td>Left</td>
<td>Occult</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>5/M/69</td>
<td>Left</td>
<td>Occult</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>6/F/71</td>
<td>Right</td>
<td>Hemorrhagic</td>
<td>1</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>7/M/83</td>
<td>Right</td>
<td>Hemorrhagic PED</td>
<td>1</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>8/M/79</td>
<td>Left</td>
<td>Predominantly classic with CRA</td>
<td>1</td>
<td>-</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>9/M/74</td>
<td>Right</td>
<td>Hemorrhagic</td>
<td>1</td>
<td>-</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>10/F/69</td>
<td>Right</td>
<td>Hemorrhagic with RPE tear</td>
<td>1</td>
<td>-</td>
<td>11</td>
<td>-</td>
</tr>
<tr>
<td>11/F/73</td>
<td>Left</td>
<td>RPE tear</td>
<td>1</td>
<td>39</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>12/F/72</td>
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<td>Hemorrhagic occult</td>
<td>1</td>
<td>39</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>13/M/73</td>
<td>Left</td>
<td>RPE tear</td>
<td>1</td>
<td>-</td>
<td>26</td>
<td>-</td>
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<tr>
<td>14/M/58</td>
<td>Right</td>
<td>Hemorrhagic with RPE tear</td>
<td>1</td>
<td>-</td>
<td>43</td>
<td>-</td>
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<tr>
<td>15/F/79</td>
<td>Right</td>
<td>Hemorrhagic with RPE tear</td>
<td>1</td>
<td>343</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>16/F/85</td>
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<td>Occult with PED</td>
<td>1</td>
<td>-</td>
<td>44</td>
<td>-</td>
</tr>
<tr>
<td>17/F/81</td>
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<td>Hemorrhagic with RPE tear</td>
<td>1</td>
<td>-</td>
<td>46</td>
<td>-</td>
</tr>
<tr>
<td>18/M/79</td>
<td>Left</td>
<td>Predominantly classic</td>
<td>1</td>
<td>64</td>
<td>48</td>
<td>-</td>
</tr>
<tr>
<td>19/M/78</td>
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<td>Occult hemorrhagic</td>
<td>5</td>
<td>249</td>
<td>209/167/125/92/50</td>
<td></td>
</tr>
<tr>
<td>20/F/76</td>
<td>Left</td>
<td>Hemorrhagic</td>
<td>1</td>
<td>-</td>
<td>53</td>
<td>-</td>
</tr>
<tr>
<td>21/M/79</td>
<td>Right</td>
<td>Occult with RPE tear</td>
<td>2</td>
<td>-</td>
<td>95/55</td>
<td></td>
</tr>
<tr>
<td>22/M/75</td>
<td>Right</td>
<td>Occult</td>
<td>1</td>
<td>-</td>
<td>57</td>
<td>-</td>
</tr>
<tr>
<td>23/F/84</td>
<td>Right</td>
<td>Hemorrhagic with PED</td>
<td>2</td>
<td>-</td>
<td>135/85</td>
<td></td>
</tr>
<tr>
<td>24/F/75</td>
<td>Right</td>
<td>Predominantly classic</td>
<td>2</td>
<td>-</td>
<td>154</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: CNV, choroidal neovascularization; CRA, chorioretinal anastomosis; PED, pigment epithelial detachment; PDT, photodynamic therapy; RPE, retina pigment epithelium.

a Patient was treated with an intravitreal triamcinolone acetonide injection 342 days before surgery.
Immunoreactivity to endostatin was detected in the RPE–Bruch membrane complex in 10 (71.4%) membranes. Endostatin was immunonegative in vessels in only 7.7% (n=1) of the specimens. Within the stroma, endostatin was expressed by fibroblast-like and inflammatory cells in 11 (78.6%) specimens (Figures 1E and 2G).

Proliferative activity varied between 6.67 and 514.08 Ki-67–expressing nuclei/mm² (median, 34.34 nuclei/mm²) (Figure 1C). Proliferating cells belonged mostly to inflammatory infiltrate within the stroma (Figure 2H).

In 12 (85.7%) CNVM, proliferative activity was less than 160 Ki-67–expressing nuclei/mm². The specimen in Figure 2H has the second highest proliferative activity among the control CNV group (511.05 nuclei/mm²).

**HISTOPATHOLOGICAL FINDINGS IN THE BEVACIZUMAB CNV GROUP**

In the bevacizumab CNV group (Figure 3A), the RPE had E-selectin in 18 (75.0%) CNVM. The EC were E-selectin immunonegative in 11 (45.8%) CNVM. Intense E-selectin staining was detected in the RPE of 6 (25%) and the EC of 8 (33.3%) CNVM. Stromal cells expressed E-selectin in 12 (50.0%) CNVM (Figures 1A and 3B). Expression of E-selectin in the bevacizumab CNV group was comparable to the control CNV group.

Immunoreactivity for ICAM-1 was detected in the RPE of 21 CNVM (87.5%) and intensely in 18 CNVM (75%). Intense ICAM-1 expression was found in the EC of 5 (20.8%) and stromal cells of 1 (4.2%) CNVM (Figures 1B and 3C). Expression of ICAM-1 was not significantly different from the control CNV group.

Leukocytes were found in all specimens (median, 271.61 cells/mm²; range, 33.50-2351.01 cells/mm²) (Figure 3D). The density of leukocytes seemed higher than in the control group, but without statistical significance (Figure 1C; P=.07).

Macrophages were present in all CNVM (median, 4661.95 cells/mm²; range, 321.01-61821.16 cells/mm²) (Figures 1C and 3E). The density of macrophages was significantly higher than that of the control CNV group (P<.001). Moderate to strong macrophage infiltration was detected within the RPE cell layer and in the perivascular area in 18 (75%) specimens and in the stroma in 17 (70.8%)
Immunoreactivity of CD68 was significantly more intense in the bevacizumab CNV group in the perivascular area (median, 3) and stroma (median, 3) than in the control CNV group (median, 1; \( P = .001 \) and median, 2; \( P = .03 \), respectively).

In the CNVM of all patients treated with bevacizumab, all vessels were lined with EC displaying Thy-1 (median, 100.0%) (Figure 3F). The percentage of Thy-1–expressing vessels was significantly higher in the bevacizumab CNV group (\( P < .001 \)).

Endostatin was intensely expressed in vessels (arrow), stromal cells (arrowheads), and retina pigment epithelium (asterisks). Many cells within the stroma expressed Ki-67 (H, arrows). Scale bars=50 \( \mu \)m.

**Figure 2.** Color fundus (A) and histologic photomicrographs (B-H) of a choroidal neovascularization membrane (CNVM) extracted without any kind of therapy before surgery. Note the massive subretinal hemorrhage (A). Sections were probed with antibodies against E-selectin (B), intercellular adhesion molecule (ICAM)-1 (C), CD45 (common leukocyte antigen; D), CD68 (E; macrophages), Thy-1 (F), endostatin (G), and Ki-67 (H). Sections were stained with 3-amino-9-ethylcarbazole red chromogen (B, C), 3-diaminobenzidine resulting in a brown chromogen (D, H), and red chromogen (E-G). Hematoxylin was used as a counterstain. B, E-selectin expression is prominent in endothelial cells (EC, arrows). ICAM-1 expression (C) was detected in the retina pigment epithelium (RPE, asterisk) and stromal cells (arrows). Leukocytes (D, arrows) and macrophages (E, arrows) were present in perivascular area and stroma. Besides Thy-1–expressing vessels (F, arrows), some Thy-1–immunonegative vessels (arrowheads) were found in the same specimen. Endostatin (G) was found to be weakly present in some vessels (arrow) whereas the RPE (asterisks) was endostatin immunonegative. H, Some proliferating cells (arrows) were present in the CNVM. Scale bars=50 \( \mu \)m.

**Figure 3.** Histologic photomicrographs of the choroidal neovascularization membranes extracted 4 days after intravitreal bevacizumab injection. Note the massive subretinal hemorrhage with pigment epithelium detachment (A). Sections were probed with antibodies against E-selectin (B), intercellular adhesion molecule (ICAM)-1 (C), CD45 (common leukocyte antigen; D), CD68 (macrophages; E), Thy-1 (F), endostatin (G), and Ki-67 (proliferating cells; H). Sections were stained with 3-amino-9-ethylcarbazole red chromogen (B, C), 3-diaminobenzidine resulting in a brown chromogen (D, H), or red chromogen (E-G). Hematoxylin was used as counterstain. Endothelial cells (arrows) and stromal cells (arrowheads) showed E-selectin (B) and ICAM-1 (C). Inflammatory infiltration with many leukocytes (D, arrows) and many macrophages (E, arrows) was detected within the perivascular area and stroma. Expression of Thy-1 was intensely expressed in vascular structures (F, arrows). G, Endostatin was intensely immunopositive in vessels (arrow), stromal cells (arrowheads), and retina pigment epithelium (asterisks). Many cells within the stroma expressed Ki-67 (H, arrows). Scale bars=50 \( \mu \)m.

Endostatin was intensely expressed in the vessels of 20 CNVM (87.3%). Stromal cells displayed endostatin in all CNVM and strongly in 21 (87.5%) membranes (Figures 1E and 3G). Endostatin was significantly stronger in the RPE-Bruch membrane complex (median, 3; range, 2-3; \( P < .001 \)) and stroma (median, 3; range, 1-3; \( P < .001 \)) of the bevacizumab CNV group than the control CNV group (median, 1.5; range, 0-3 and median, 1; range, 0-3, respectively).

Proliferative activity (median, 204.33 cells/mm\(^2\); range, 3.61-1886.135 cells/mm\(^2\)) in the bevacizumab CNV group was significantly higher than that in the control CNV group (\( P = .007 \)) (Figures 1C and 3H).
immunohistologic findings in the bevacizumab CNV group did not show major differences based on either timing or number of bevacizumab injections (Table 3). Based on previous application of PDT, only E-selectin expression showed a major difference, being significantly stronger in the EC of patients in the bevacizumab CNV group treated with PDT previously (n=4) than those in the bevacizumab CNV group without any previous PDT (n=20) (Table 3).

**COMMENT**

Vascular endothelial growth factor is expressed in the choroid of human eyes, and polarized secretion of VEGF in the RPE is vital for choriocapillaris development and maintenance. In AMD, various factors such as free radicals or complement components may upregulate VEGF expression. Vascular endothelial growth factor, chemoattractant for inflammatory cells, activates monocytes and macrophages and induces expression of ICAM-1 and E-selectin on vascular EC to facilitate adhesion and migration of inflammatory cells. Macrophages synthesize the cytokines IL-1 and TNF-α, which enhance ICAM-1 expression in EC and in the RPE, and induce inflammatory cell infiltration further. There is a closed but amplifying circuit between VEGF and inflammatory cells, because leukocytes and macrophages can produce VEGF themselves and also induce VEGF production in the RPE through IL-1 and TNF-α in human CNVM. Vascular endothelial growth factor, in turn, stimulates growth and maintenance of the CNVM. Because VEGF and inflammation seem to closely interact with each other, VEGF inhibition by bevacizumab might influence, or possibly inhibit, the inflammatory activity in CNV.

In the specimens treated with bevacizumab, expression of ICAM-1 and E-selectin was comparable to that of the control CNV cases without previous therapy. However, density of leukocytes, and especially of macrophages, was higher in the bevacizumab CNV group than in the control CNV group. Consequently, the percentage of Thy-1–expressing vessels, a mirror of TNF-α and IL-1 activity, was also significantly higher. Macrophages, reported to be present in 60% of the surgically excised CNVM, seem to play a causative role in neovascular AMD pathogenesis. In aged RPE cells, accumulation of oxidation products seems to induce angiogenic and chemotactic factors like monocyte chemoattractant protein 1. In healthy human eyes, recruited macrophages may have a physiological role in removing the accumulated subretinal debris. However, excess oxidized lipids in the eyes of patients with AMD were suggested to upregulate the release by macrophages of proteolytic enzymes that damage the Bruch membrane and contribute to CNV development. Macrophages, in addition to stimulating VEGF, can induce EC proliferation and migration through the release of cytokines, proteases, and growth factors. Therefore, experimental studies for potential new antiangiogenic treatment strategies target the complement system, ICAM, macrophages, monocytes, and TNF. These were shown to inhibit leukocyte infiltration and VEGF expression and, therefore, inhibit CNV development or progression in experimental studies. In experimental models, early intravitreal injection of VEGF-A antibody after CNV induction was shown to inhibit CNV and choroidal macrophage in-
filtration through suppression of monocyte chemoattractant protein 1 expression in the RPE-choroid complex. Hence, anti-VEGF drugs might be expected to have an anti-inflammatory effect.

Our results were truly unexpected. Inhibition of VEGF with bevacizumab was related to a prominent inflammatory reaction, instead of an anti-inflammatory effect, in comparison to the control CNV group. This might be for several reasons.

First, the Fc portion of bevacizumab in CNV may stimulate a cellular immune response through Fc receptors in immune cells. Bevacizumab was shown to penetrate the retina in an experimental study. Anatomical defects in the Bruch membrane created by proteolytic enzymes and CNV itself might facilitate the passage of bevacizumab molecules through the RPE to the CNVM in the eyes of patients with AMD.

Second, VEGF acts as a survival factor for EC by inhibiting apoptosis. Deprivation and/or inhibition of VEGF was shown to induce vascular regression through EC apoptosis, especially in neovascular vessels. During vascular regression, macrophages regulate EC and pericyte apoptosis in capillaries. Leukocytes release human neutrophil peptides that are proapoptotic for EC. Hence, endostatin was shown to inhibit CNV. Furthermore, endostatin is released and activated. In our series, timing of the bevacizumab injection prior to surgery varied between 1 and 154 days. Four of the 24 patients were treated with 2 to 5 bevacizumab injections preoperatively. However, immunohistologic findings in the bevacizumab CNV group did not show major differences based on timing or number of injections. Four of the patients were treated with PDT 39 to 343 days before bevacizumab injection. Of the antigens investigated, only E-selectin was found to be significantly more intense in the EC of the bevacizumab CNV patients treated previously with PDT. However, our previous studies have shown that immunohistopathological findings dramatically change early (3 days) after PDT when compared with either longer intervals following PDT or the control CNV group. The implications of PDT-bevacizumab combination therapy on inflammation and angiogenesis, therefore, need to be evaluated in a higher number of samples excised early after combination therapy.

To our knowledge, this is the first clinicopathological study evaluating inflammation and proliferation in human CNV treated with an anti-VEGF agent. To interpret the results properly, the following limitations need to be taken into account: first, histopathological specimens reflect only a time window within a dynamic process. Therefore, we selected homogenous control and treatment groups that were comparable in their clinics. Second, in contrast to experimental studies, time between treatment and tissue acquisition varies. Nevertheless, data retained by histopathological examination of human samples may be closer to the truth than any data collected by in vitro or in vivo experiments.

The results of this study, revealing high inflammatory and proliferative activity, were truly unexpected and appear to be, to some extent, paradoxical to the inhibition of angiogenesis. The question is how to interpret this data in view of promising clinical results related to a decrease in disease activity following intravitreal injection of bevacizumab. There is growing knowledge that inflammatory reactions exert not only proangiogenic activities, but are also involved in vascular regression and angiogenesis inhibition. We assume that a similar process may be reflected in our histopathological results. Our results reveal enhanced expression of the endogenous angiogenesis inhibitor endostatin in these inflammatory active bevacizumab CNVM. Still, our understanding of treatment modalities and combination therapies needs to be further evaluated to implement the optimal treatment
strategies and protocols for neovascular AMD. High inflammatory and proliferative activity as well as enhanced endostatin expression in bevacizumab-treated CNV should be considered when combination therapies with bevacizumab are planned.\textsuperscript{11,82,83}

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**Author Affiliations:** University Eye Hospital, Centre for Ophthalmology, Eberhard-Karls University, Tübingen (Drs Tatar, Yoeruek, Szurman, Bartz-Schmidt, and Grisanti); Department of Pathology, University of Tübingen (Ms Adam), Augenklinik der Städtischen Kliniken, Frankfurt am Main (Dr Eckardt); and Augenzenrum Recklinghausen, Recklinghausen, Germany (Dr Scharioth); Laboratory of Visual Physiology, National Institute of Sensory Organs, Tokyo, Japan (Dr Shinoda); Algemeen Ziekenhuis Sint Augustinus Hospital, Department Achtsegment, Antwerp, Belgium (Drs Boeyden and Claes); and Department of Ophthalmology, Sacro Cuore Hospital, Negrar, Italy (Dr Peri tile). Dr Grisanti is now with the Department of Ophthalmology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany (Salvatore.Grisanti@uk-sh.de).

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